Fast Analysis of Therapeutic Monoclonal Antibodies, Fragments, and Oxidation Variants Using a Super-Macro Porous Reversed-Phase Column Coupled with an Orbitrap Mass Spectrometer

Poster Note 64398

Shanhua Lin,[1] Terry Zhang,[2] Hongxia Wang,[2] Jonathan Josephs,[2] and Xiaodong Liu[1]
[1]Thermo Fisher Scientific, Sunnyvale, CA; [2]Thermo Fisher Scientific, San Jose, CA

Overview

Purpose: To achieve high resolution separation of mAb fragments and oxidized fragments.

Methods: Reverse phase separation of mAb and mAb fragments using Thermo Scientific™ MAbPac™ RP column coupled with Thermo Scientific™ Q Exactive™ Plus Orbitrap™ mass spectrometer.

Results: LC, HC, Fc, Fab, scFc and $F(ab')_2$ are successfully separated using a 10-min gradient. Mass of oxidized HC is fully resolved from non-oxidized HC.

Introduction

The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increased demand for targeted treatments. Therapeutic mAbs, such as Rituxan, Herceptin, Remicade, and Avastin, are mostly produced from mammalian cells. These biological products are heterogeneous due to post-translational modifications. Additional modifications such as oxidation can be introduced during the manufacturing process. A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing processes.

There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of mAbs using high resolution mass spectrometers. The most commonly employed LC/MS method is to desalt mAbs via reversed phase liquid chromatography and perform an MS analysis. Further MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), Fc, and Fab can quickly reveal the location as well as nature of the modification.

In the current study, we are presenting a fast separation method for mAb fragments from Rituxan, Herceptin, Remicade, and Avastin using a novel supermacro porous reversed phase (SMP RP) column. The mAb fragments were then generated by subsequent DTT reduction, papain digestion, or ideS protease digestion. Baseline separation of heavy chain and light chain, Fab and Fc fragments, scFc and $F(ab')_2$ was achieved in all cases using a 10-min gradient with water/acetonitrile/TFA mobile phases. Using an orbitrap mass spectrometer accurate masses of mAb fragments were measured and the presence of oxidation variants was detected. The fast LC/MS approach described here can be generally applicable to mAb variant characterization.

Methods

Chemicals and reagents

Papain was purchased from Sigma-Aldrich. FabRICATOR® (IdeS) Protease was purchased from Genovis. Therapeutics monoclonal antibodies Rituxan®, Herceptin®, Remicade® and Avastin® were kind gifts from Professor Mark Avdalovic at California National Primate Research Center for Respiratory Disease, University of California, Davis.

Columns

MAbPac RP 4 µm, 3 × 50 mm, P/N 088645

LC instruments

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

SRD-3400 Solvent racks with degasser

HPG-3400RS Biocompatible Binary Rapid Separation Pump

WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler

TCC-3000RS Rapid Separation Thermostatted Column Compartment

VWD-3400RS Rapid Separation Variable Wavelength Detector

Mobile phases:

Mobile phase A: H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)

Mobile phase B: MeCN/ H_2O/FA/TFA (90:9.88 :0.1:0.02 v/v/v/v)

Reduction:

Reduction of inter-chain disulfides in a mAb (4 mg/mL) was achieved by incubation of mAb with 20 mM DTT at 37 °C for 30 min.

Papain digestion:

The digestion was carried out by incubating mAb (2 mg/mL) with papain (0.04 mg/ml) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA and 5 mM Cysteine buffer at 37 °C for 4 hours.

IdeS digestion:

IdeS protease was added at 1 unit enzyme per 1 µg of mAb ratio. The digestion was carried out in 50 mM sodium phosphate, 150 mM NaCl (pH 6.6) buffer at 37 °C for 30 min.

Preparation of H_2O_2 oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2X oxidation buffer (360 mM sodium chloride, 10 mM sodium acetate, pH 5.0). Then add H_2O_2 to a final concentration of 0.01% (v/v) and incubate the sample for 24h at room temperature.

Mass Spectrometry conditions

The Q Exactive Plus mass spectrometer was used for this study. Intact mAb or mAb fragments were analyzed by ESI-MS. H-ESI II probe was used. The resolution was set at 17.5 k (FWHM at *m/z* 200) see Table 1.

Data processing

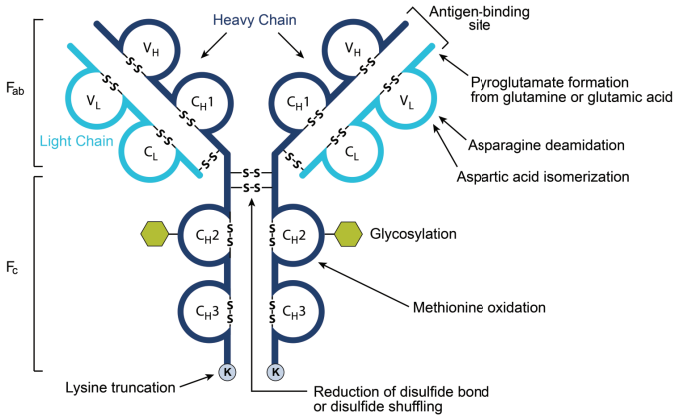
Full MS spectra of intact mAbs and mAb fragments were analyzed using Thermo Scientific™ Protein Deconvolution™ software 2.0 that utilizes the ReSpect algorithm for molecular mass determination.

TABLE 1. MS conditions.

Instrument Conditions	mAb and mAb Fragments
Mass range	m/z 1,000–4,000
Spray voltage	3.9 kV
Sheath gas	45 arb. units
Auxiliary gas	15 arb. units
Capillary temperature	320 °C
S-lens level	55
In-source CID	40 eV
Microscans	10
AGC target	3×10^6
Maximum IT	200 ms
Resolving power	17,500
Probe temperature	300 °C

Thermo SCIENTIFIC

FIGURE 1. Structure of IgG and typical forms of heterogeneity.

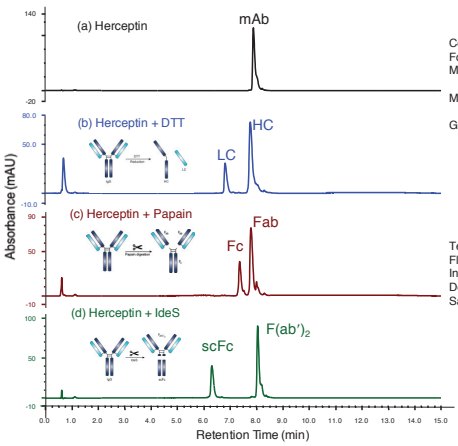


Results

Monoclonal Antibody Fragment Analysis

Monoclonal antibodies are heterogeneous (Figure 1). Comprehensive analysis of mAb post-translational modifications, such as deamidation, C-terminal lysine truncation, *N*-terminal pyroglutamation, methionine (Met) oxidation, and glycosylation, requires complete digestion of the mAbs and sequencing of all the peptides. However, "peptide mapping" is time consuming. A simpler and direct way to analyze the mAb variants and locate the modifications is to measure mAb fragments. Light chain (LC) and heavy chain (HC) are generated by the reduction of mAb, Fc and Fab fragments are generated by papain digestion. scFc and $F(ab')_2$ fragments are generated by IdeS digestion. Figure 2 shows the analysis of Herceptin intact molecule and Herceptin mAb fragments. LC and HC (Figure 2b), Fc and Fab (Figure 2c), scFc and $F(ab')_2$ (Figure 2d) are baseline separated using a MAbPac RP column with a 10-min gradient. Similar experiments have been carried out for Rituxan, Remicade, and Avastin. In all cases, mAb fragments have been successfully separated (data not shown).

FIGURE 2. Analysis of Herceptin and Herceptin fragments using MAbPac RP. (a) Herceptin; (b) Herceptin LC and HC; (c) Herceptin Fc and Fab fragments; (d) Herceptin scFc and $F(ab')_2$ fragments.

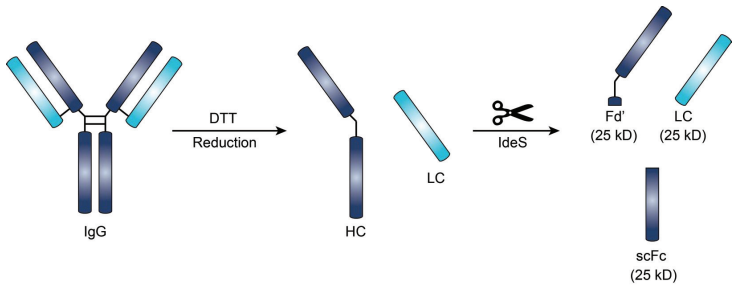


Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88:0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90:9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	5 µL
Detection:	UV (280 nm)
Sample:	(a) Herceptin (5 mg/mL) (b) Herceptin + DTT (4 mg/mL) (c) Herceptin + Papain (2 mg/mL) (d) Herceptin + IdeS (2 mg/mL)

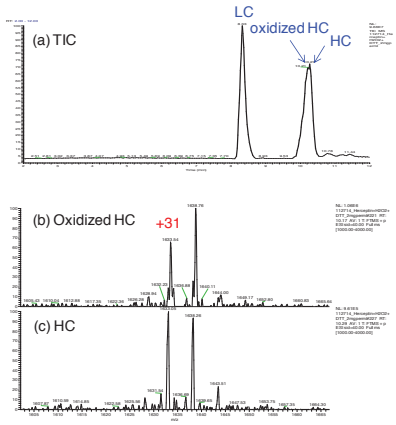
FIGURE 3. mAb reduction and IdeS digestion flowchart.



Monoclonal Antibody Oxidation Analysis

Met oxidation is one of the critical quality attributes required to be closely monitored. The two Met residues in the CH2-CH3 domain interface of recombinant humanized and fully human IgG1 antibodies were found susceptible to oxidation [1]. It is desirable to monitor the progress of the Met oxidation without complete digestion of mAb. A workflow was designed to first reduce mAb and then further digest it with IdES resulting in smaller (25 kDa) fragments (Figure 3). Figure 4a shows that oxidized HC and non-oxidized HC can be barely separated chromatographically. However, the high resolution Orbitrap instrument can clearly resolve the oxidized (Figure 4b) and non-oxidized HC (Figure 4c) at *m/z* 1633.54 and 1633.06 respectively. Further digestion of the HC by IdeS resulted in two smaller fragments: scFc and Fd′. Figure 5a shows the baseline separation of scFc, LC, and Fd′. In addition, oxidized and non-oxidized scFc fragments are better separated than the oxidized and non-oxidized HC. The +10 charge state of the oxidized scFc and non-oxidized scFc are shown in Figure 5b (at *m/z* 2525.60) and in Figure 5c (at *m/z* 2524.08). Data collected using 280K resolution shows isotopic ally resolved oxidized scFc (figure 6a) and non-oxidized scFc (figure 6b) at +16 charge state. Separation of these oxidized and non-oxidized fragments can be improved by increasing the mobile phase TFA concentration from 0.02% to 0.1% (Figure 7).

FIGURE 4. LC/MS analysis of Herceptin/trastuzumab LC and HC. (a) Total ion current (TIC); (b) mass spectrum of oxidized HC; (c) mass spectrum of non-oxidized HC.

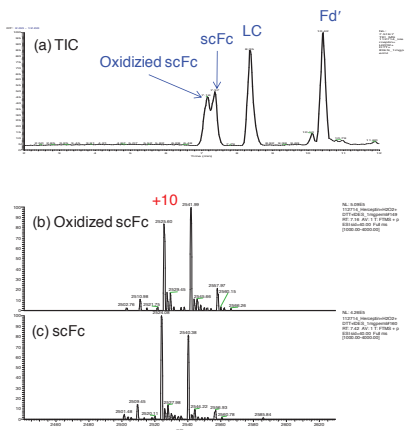


Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	75	25
1.0	75	25
11.0	63	37
12.0	63	37
14.0	75	25
15.0	75	25

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
MS Detection:	positive-ion mode
Mass spec:	Q Exactive™ Plus
Sample:	oxidized Herceptin, reduced by DTT (2 mg/mL)

FIGURE 5. LC/MS analysis of Herceptin/trastuzumab scFc LC, and Fd′. (a) Total ion current (TIC); (b) mass spectrum of oxidized scFc; (c) mass spectrum of non-oxidized scFc.



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	75	25
1.0	75	25
11.0	63	37
12.0	63	37
14.0	75	25
15.0	75	25

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
MS Detection:	positive-ion mode
Mass spec:	Q Exactive™ Plus
Sample:	Oxidized Herceptin, reduced by DTT and digested by IdeS (1 mg/mL)

FIGURE 6. High resolution MS for oxidized Herceptin/trastuzumab scFc analysis. (a) oxidized scFc; (b) scFc.

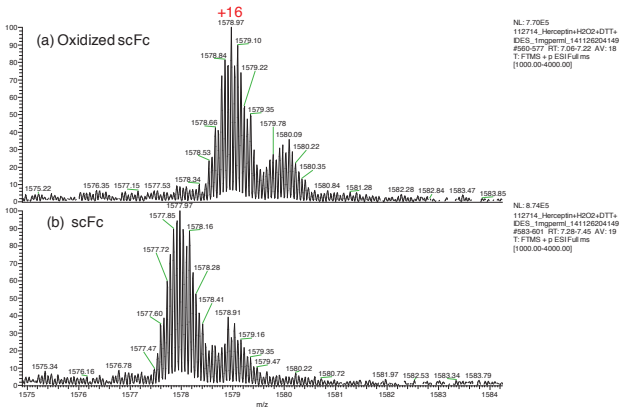
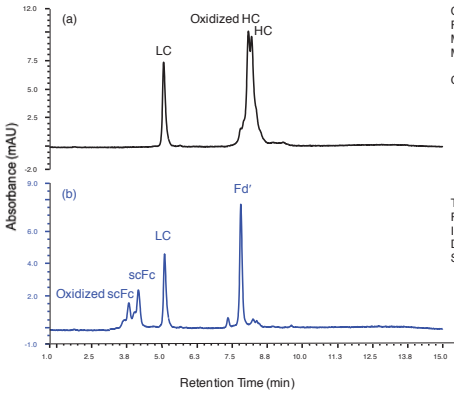


FIGURE 7. Separation of Herceptin/trastuzumab fragments with mobile phases containing 0.1% TFA. (a) Herceptin LC and HC; (b) Herceptin scFc, LC, and Fd′.



Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	70	30
1.0	70	30
11.0	60	40
12.0	60	40
14.0	70	30
15.0	70	30

Temperature:	80 °C
Flow rate:	0.5 mL/min
Inj. volume:	2 µL
Detection:	UV (280 nm)
Sample:	(a) Herceptin + DTT (2 mg/mL) (b) Herceptin + DTT + IdeS (1 mg/mL)

Conclusions

- mAb LC, HC, Fc, Fab, scFc and F(ab′)$_2$ fragments are successfully separated using MAbPac RP column and a 10-min gradient.
- Mass of oxidized HC is fully resolved from non-oxidized HC using Q Exactive Plus mass spectrometer.
- Separation of oxidized versus non-oxidized fragments is improved using 0.1% TFA-containing mobile phases.

References

1. Liu H., Gaza-Bulseco G., and Zhou L. Mass Spectrometry Analysis of Photo-InducedMethionine Oxidation of a Recombinant Human Monoclonal Antibody, J. Am. Soc. Mass. Spectrom., 2009, 20, 525-528

Acknowledgements

We would like to thank Professor Dr. Mark V. Avdalovic, from the California National Primate Research Center for Respiratory Disease, University of California, Davis, for supplying the monoclonal antibodies.

© 2015 Thermo Fisher Scientific Inc. All rights reserved. FabRICATOR is a trademark of Genovis AB. Rituxan is a trademark of Biogen Idec, Inc. Herceptin and Avastin are trademarks of Genentech, Inc. Remicade is a trademark of JanssenBiotech, Inc. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.

www.thermoscientific.com

©2015 Thermo Fisher Scientific Inc. All rights reserved. ISO is a trademark of the International Standards Organization. FabRICATOR is a trademark of Genovis AB. Rituxan is a trademark of Biogen Idec, Inc. Herceptin and Avastin are trademarks of Genentech, Inc. Remicade is a trademark of JanssenBiotech, Inc. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Thermo Fisher Scientific, San Jose, CA USA is ISO 13485 Certified.

ISO 13485

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 45 453 9100
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Spain +34 914 845 965
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

PN64398-EN 0615S